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The underlying reason for failure to cure patients diagnosed with breast cancer is the presence of micrometastases. The stimulation of anti-tumor immune responses represents one of the most effective ways to treat low tumor burdens that are microscopically or clinically occult. The objective of our proposal is to determine whether a poliovirus replicon vaccine strategy engineered to express human CEA or the HER2/neu oncogene can induce systemic immunity and eradication of micrometastases.

During the second year of the project, we have compared the expression of CEA from replicons which encode different versions of CEA (i.e. with and without signal sequence or membrane anchor region) using two different replicons. Mice given the replicons encoding the signal minus CEA generated an antibody response against CEA. Tumor challenge of these animals is ongoing. We have constructed replicons which encode the extracellular domain of HER2/neu. Expression was confirmed using antibodies to HER2/neu and the analysis of the immune response in mice is ongoing. The results of these studies provide essential preclinical observations that will be relevant to the starting of human breast cancer trials targeted against cells that express CEA or HER2/neu.

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INTRODUCTION

The frequency of micro-metastatic cancer deposits has correlated with a number of factors at the time of primary therapy of breast cancer including regional lymph node metastasis, size of primary tumor, tumor differentiation and certain molecular aspects of individual tumors including aneuploidy, DNA synthesis (S phase) and presence or absence of amplified gene expression for c-myc, p53, or HER2/neu. The use of systemic hormonal therapy, chemotherapy or combined adjuvant therapy directed at these micro-metastases has produced a reduction in metastatic relapse rate and improved survival. These effects are modest, and the majority of patients with micro-metastasis still have disease recurrence and ultimately die of metastatic breast cancer. **Thus, it is clear that additional strategies to eradicate micro-metastasis at the time of initial diagnosis is a major priority in breast cancer therapy research.** It should also be noted that our inability to detect occult micro-metastases means that patients with and without micro-metastases will be treated. The strategy should have low toxicity, ease of administration and cost-effectiveness (low cost).

Multiple animal model studies (6, 7) have demonstrated that immune response to tumor associated antigens can have dramatic antitumor effects, but such treatments strategies rapidly lose efficacy as progressive tumor growth occurs (progressive time after tumor implantation or metastasis). Thus, the induction of an immune response to tumor associated antigens in humans is likely to have limited success in patients with obvious metastases and its optimal application would be at the time of occult micro-metastasis as an adjunct to primary therapy. Active specific immunotherapy to enhance host immune response to tumor associated antigens has been called "vaccine" therapy although this application does not fit the strict (narrow) definition that entails prevention of disease rather than therapy of an existing disease. It is the purpose of this project to define a novel strategy to enhance antitumor response to tumor-associated antigens, leading to the development of therapeutic vaccines.

A variety of studies have demonstrated that immunization with tumor preparations can produce antitumor immune responses in animal models (4, 5, 12). Similar studies in man have shown antitumor effects in patients with melanoma (3, 10)and other tumors (9). The ability to isolate and clone putative tumor antigens provides the opportunity to utilize more defined reagents and to allow analysis of specific immune responses in guiding the design of active immunotherapy trials. A variety of potential targets include CEA, HER2/neu, MUC-1, MAGE 1, mutated RAS, mutated p53, etc. (7). A number of genetically engineered cancer vaccines utilizing cloned tumor associated antigens in vaccinia virus constructs or with adjuvants are undergoing clinical trials (1). We believe that this approach represents a fertile and novel new technology, and that we are just beginning to identify potential tumor associated antigens (and their genes) which will be applicable to novel strategies for enhancement of anti-tumor immune responses in animal models and man.

Poliovirus Replicons to Express Foreign Genes

The proposed experiments are based on the use of poliovirus as an expression vector for proteins to deliver antigens to immunoreactive sites of the immune system. Poliovirus is attractive for use as a vector for cancer vaccines for several reasons. First, it is an RNA virus with no DNA intermediates in replication. Thus, we can formulate vaccines with oncogenes (e.g. HER2/neu) without concern for cellular transformation (8). Second, my laboratory has developed a unique vector system based an poliovirus for the expression of foreign genes. To date, we have constructed poliovirus genomes encoding foreign proteins, referred to as replicons, for over twenty different genes. We have also developed the procedure in which we can complement these replicons by providing the capsid protein *in trans*. We are able to generate stocks of encapsidated replicons which encode foreign proteins which, upon infection of cells, express this recombinant protein. We have demonstrated that administration of replicons alone to experimental animals results in production of an immune response to the foreign protein (11).

BODY OF THE PROPOSAL

The Specific Aims of the proposal have not changed and are as follows:

1 To construct poliovirus replicons which express native and truncated CEA proteins (including secreted and non-secreted molecules (months 1-18).

2. To characterize and optimize the immune response to CEA elicited by both oral and parenteral administration of such vaccines in mice (months 4-24).

3. To test the ability of such poliovirus - replicon CEA vaccines to generate antitumor effects as measured by resistance to tumor challenge in a syngeneic murine CEA expressing breast cancer model (months 12-36).

4. To test the therapeutic effects of such vaccines in the eradication of breast cancer micro-metastasis in a syngeneic, spontaneously metastasizing CEA positive breast cancer model (months 18-48).

During the first 24 months, we have made progress towards completion of the designated experiments described in Specific Aim 1 and 2. To date, we have constructed three replicons which encode the CEA protein: 1) the complete CEA gene protein minus the signal sequence (Sig-CEA), 2) the complete CEA gene, and 3) the CEA gene lacking the carboxyterminal 27 amino acids to prevent membrane anchorage (anchor-CEA or TM-CEA). These genes have been positioned into different replicons, one encoding the VP4 protein of poliovirus and one without the VP4 protein. We have characterized the expression of CEA from all replicons. We present the expression from the Sig-CEA and the anchor-CEA (Figure 1A).

Based on our studies, two replicons were chosen for further analysis. The first replicon contains the complete CEA gene minus the signal sequence and without VP4 sequence (Sig-CEA) (2). The second replicon is the anchor-CEA (VP4-TM-CEA).

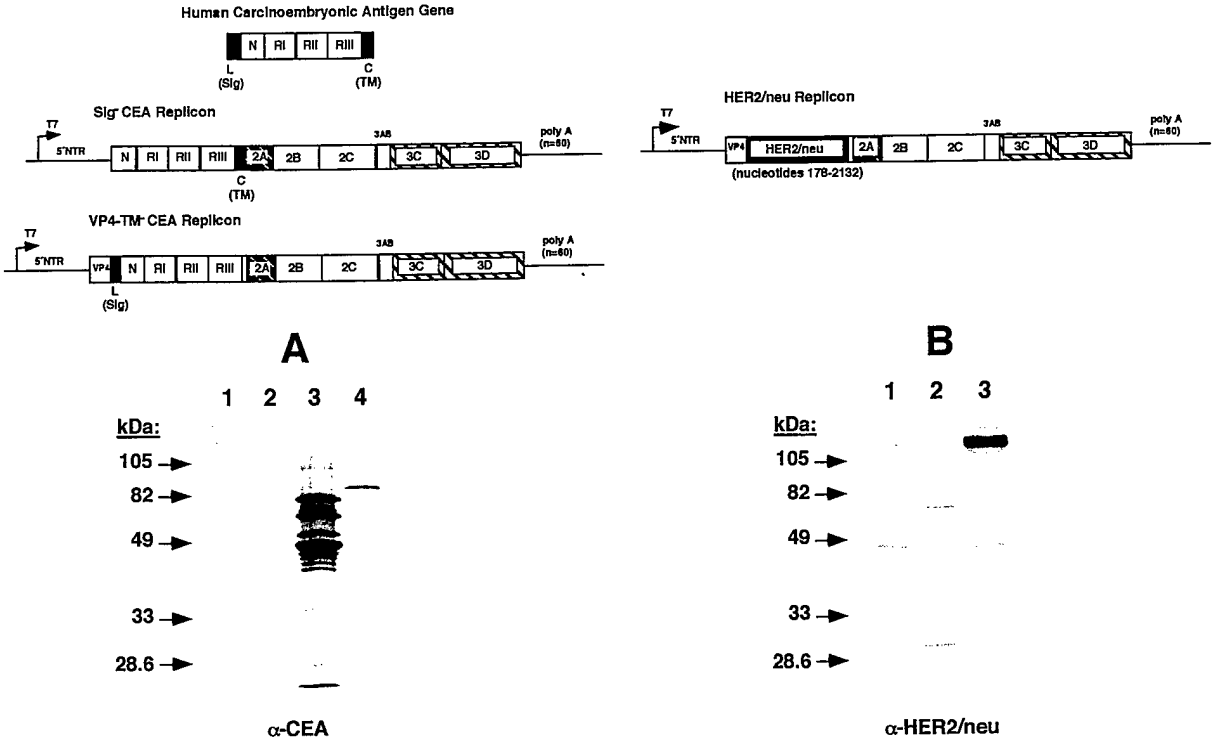

Figure 1. Expression of CEA and HER2/neu proteins from replicons. Panel A. HeLa H1 cells were either mock infected (lane 1) or infected with replicons expressing tetanus toxoid C-fragment (lane 2), CEA/Sig- (lane 3), or VP4-CEA/TM- (lane 4). Proteins were metabolically radiolabeled with 35S-methionine/cysteine and immunoprecipiated with a monoclonal antibody specific hor human CEA (Col-1). Several immunoreactive proteins (from 45-80 kDa) were detected from cells infected with the CEA/Sig- replicon (lane 3), some of which likely represent products of proteolytic digestion of the primary CEA product. One predominant CEA-specific product (approximately 95 kDa) was detected from cells infected with the VP4-CEA/TM- replicon (lane 4). **Panel B.** Hela H1 cells were either mock infected (lane 1) or infected with replicons expressing tetanus toxoid C-fragment (lane 2) or HER2/neu (lane 3). Proteins were metabolically radiolabeled with 35S-methionine/cysteine and immunoprecipitated with a monoclonal antibody that recognizes HER2/neu. Two high molecular weight immunoreactive proteins with similar mobilities were detected from cells infected with the HER2/neu replicon (lane 3).

In a related set of studies, we have extended our analysis to other another gene relevant to breast cancer, the HER2/neu oncogene. We have constructed a replicon which encodes the extracellular domain (ECD) of HER2/neu. The replicon expressed a protein that was immunoprecipitated with antibodies specific for the extracellular domain of HER2/neu (Figure 1B).

Analysis of the immunogenicity of encapsidated replicons encoding CEA or HER2/neu.

We have made significant progress analyzing the immunogenicity of the replicons encoding CEA. Two experimental approaches were explored. In the first, we used direct injection of the unencapsidated replicon

RNA, generated from *in vitro* transcription, into C57BL/6 mice. Construction for immunization included poliovirus replicons encoding CEA lacking the carboxyterminal 27 amino acids to prevent membrane anchorage yielding a secretory product (polio/anchor (-)CEA) and CEA lacking the ER signal sequence (polio/sig (-) CEA).

In a pilot study, we immunized groups of 10 to 12 mice with 50 μg doses of each unencapsidated RNA by i.m. injection every three weeks times for a total of five injections followed by challenge with syngeneic, CEA-expressing colon carcinoma cells two weeks after the last immunization. A group of 12 naive mice were tumor challenged as controls. As shown in Figure 2, tumors developed in 8 of 12 naive mice, 0 of 10 mice immunized with polio/sig (-) CEA, and 1 of 10 mice immunized with polio/anchor (-) CEA. No evidence of anti-CEA antibody response was observed among animals receiving unencapsidated replicon RNA, despite near complete protection against tumor challenge.

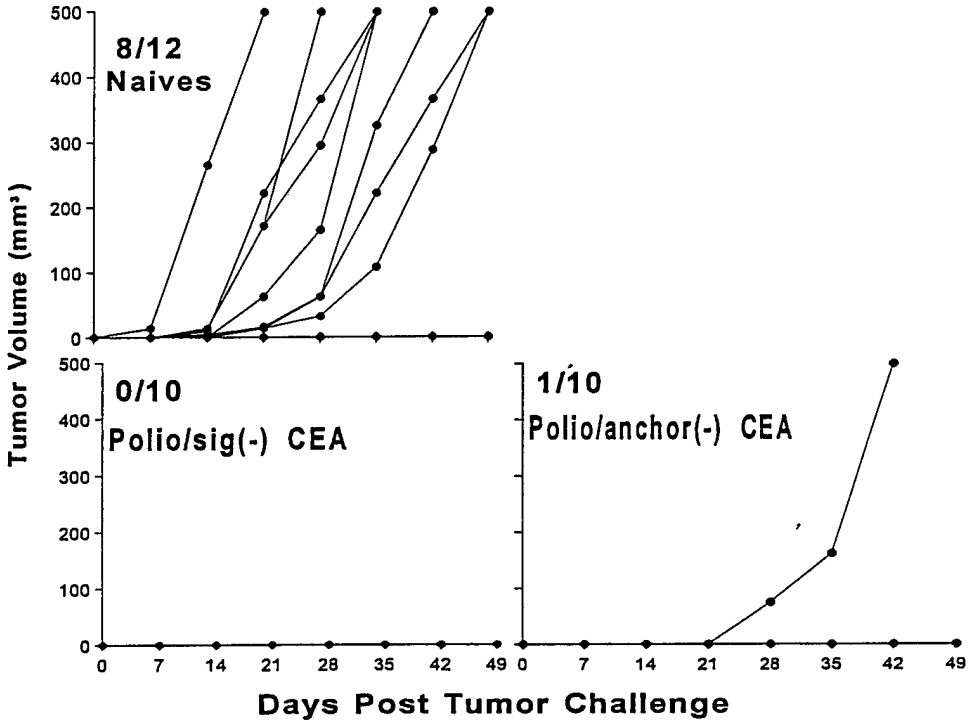

Figure 2: Tumor protection elicited by immunization with unencapsidated poliovirus RNA encoding various forms of CEA.

In the second approach, we used transgenic mice which express the human receptor for poliovirus. Previous studies have found that these mice are susceptible to poliovirus infection when given intramuscularly (13). Furthermore, we have shown intramuscular administration of replicons encoding foreign proteins results in specific immune responses to the encoded protein (2, 11). We have extensive experience with the C57BL/6 derived adenocarcinoma model stably expressing human CEA by retroviral transfection (MC38-CEA). We initially performed a series of experiments to demonstrate that the MC38-CEA model translates into human poliovirus receptor transgenic mice which were derived from C57BL/6 x CBA mice. Inoculation of 2.5 $x10^5$ MC38-CEA cells into the flank of human poliovirus receptor transgenic mice by subcutaneous injection produces measurable tumor outgrowth in greater than 90% of mice within 4 weeks and these animals go on to die as a result of tumor burden.

In a pilot experiment, groups of ten mice transgenic for the human poliovirus receptor received 10^7 infectious units of encapsidated polio replicons encoding either CEA with the ER signal sequence deleted [Sig (-) CEA] or the C fragment of tetanus toxin as a negative control antigen by intramuscular injection days 1,22, 43 and 69. As shown in Table 1, among mice receiving replicons encoding Sig (-)CEA, anti-CEA antibody responses were observed in 5 of 10 mice 7 days after the third immunization and in 10 of 10 mice 7 days after the fourth immunization. No evidence of anti-CEA antibody response was observed among mice receiving replicons encoding tetanus toxin as a negative control. These animals have now been challenged with MC38-CEA tumor cells with monitoring of tumor outgrowth ongoing.

Table1: Anti-CEA antibody response among polio receptor transgenic mice receiving polio replicons by I.M. injection.

	Tetanus Toxoid Replicons		Sig (-) CEA Replicons	
Mouse	**Day 50**	**Day 77**	**Day 50**	**Day 77**
1	2	0	23	1,000
2	5	0	230	3,300
3	2	0	39	16,000
4	10	41	11	5,300
5	15	0	33	250
6	5	0	60	1,200
7	12	0	280	17,000
8	8	19	47	8,900
9	10	0	89	1,500
10	8	40	119	1,300

Values are ng of ^{125} I-CEA bound per ml of sera with values $\geq$ 50 representing a positive result.

To further extend our studies on CEA, analysis of the immune responses to encapsidated replicons encoding CEA delivered by intraperitoneal injection are also underway. Finally, we have also initiated studies of encapsidated replicons encoding CEA co-delivered with wild-type Sabin poliovirus by i.m. or i.p. injection in an attempt to facilitate a limited number of infection cycles of the CEA encoding replicons. Previous studies from our laboratory have demonstrated that replicons encoding different antigens were immunogenic when administered in this way (11).

In a related series of experiments, we have also immunized the transgenic mice with the replicons encoding HER2/neu. The mice have now been immunized and boosted once. From our initial analysis, we have detected antibodies to both poliovirus as well as HER2/neu. Following evaluation of the immune response, we anticipate challenging these animals with tumors encoding HER2/neu (the generation of these tumor cells by transfection of a plasmid encoding HER2/neu is underway).

In the next period, we will continue the evaluation of the animals immunized with the encapsidated replicons encoding CEA. Based on our preliminary studies with RNA injection, we anticipate that these animals will be protected against the tumors expressing CEA. As a corollary to these experiments, we will also evaluate those animals immunized with HER2/neu for resistance to tumors expressing HER2/neu. We have also begun immunization of animals with CEA replicons via mucosal routes (nasal, oral, and intraperitoneal). We will also analyze the immunized mice for cell mediated immune responses (proliferation to CEA and cell mediated immunity (CTL)). The mice will be evaluated for an antibody response to CEA prior to challenge with the tumor expressing CEA. Finally, we will initiate experiments in the latter half of the year relating to Specific Aim 4 to test the therapeutic effects of such vaccines in animals. For these studies, we will use the spontaneously metastasizing CEA positive breast cancer model as detailed in the original proposal.

CONCLUSION

The proposed studies for CEA are progressing on schedule. As planned, we have now defined the appropriate replicon for expression of CEA and demonstrated the immunogenicity of this replicon when given via the intramuscular route. In preliminary experiments, we have determined that immunization of these animals with replicon RNA alone resulted in the generation of a slight but significant anti-tumor response. Ongoing experiments utilizing animals immunized with encapsidated replicon which have demonstrated an immune response to CEA should provide us with further substantiation that administration of replicons induces a protective immune response. We have extended our studies to different breast cancer related oncogene such as HER2/neu. Replicons encoding this oncogene have been constructed and evaluated for expression. Evaluation of the efficacy of these replicons in animals is underway.

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